The Journal of Experimental Biology 202, 3135–3143 (1999)
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JEB2126



# REACTIVE OXYGEN INTERMEDIATE PRODUCTION BY OYSTER HEMOCYTES EXPOSED TO HYPOXIA

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*Accepted 13 September; published on WWW 28 October 1999*

## Summary

**Oysters are frequently exposed to severely hypoxic conditions, especially during summer months. During the summer, there are also large numbers of disease-related oyster mortalities. This research was conducted to determine whether exposure to environmental hypoxia reduces the ability of oyster hemocytes to produce reactive oxygen intermediates (ROIs), an important part of their defense system. Oysters of the species *Crassostrea virginica* were held in normoxic ($P_{O_2}$=20.0–20.7 kPa, pH 7.8–8.0) and hypoxic conditions ($P_{O_2}$=4.0–6.7 kPa, pH 7.1–7.4). *In vivo* hemolymph variables ($P_{O_2}$, $P_{CO_2}$ and pH) were measured after both 1 hour and 2 days in each treatment to determine the appropriate environment for subsequent hemocyte experiments. Production of reactive oxygen intermediates by hemocytes was measured using luminol-enhanced chemiluminescence (CL). During CL tests, hemocytes were held under the following conditions: air ($P_{O_2}$=20.7, $P_{CO_2}$<0.07, pH 7.6), *in vivo* hemolymph conditions of normoxic oysters ($P_{O_2}$=5.2, $P_{CO_2}$=0.27, pH 7.6), and *in vivo* hemolymph conditions of hypoxic oysters ($P_{O_2}$=1.47, $P_{CO_2}$=0.53, pH 7.1). Production of ROIs under hypoxic conditions was 33 % of that under normoxia. This decrease was the result of specific and independent effects of lower oxygen levels and decreased pH. It was not due to any direct effect of $CO_2$.**

Key words: chemiluminescence, hemocyte, hypoxia, oyster, reactive oxygen intermediate.

## Introduction

Organisms living in estuaries are often subjected to harsh living conditions associated with frequent changes in their aquatic environment. Hypoxia is one condition that can be particularly stressful. Periods of low oxygen are especially common during late summer months in the estuarine environment and may be severe (Breitburg, 1990, 1992; Cochran and Burnett, 1996). Benthic and sessile organisms, such as oysters, are particularly vulnerable to hypoxia. Furthermore, low oxygen is not the only condition that oysters must contend with during periods of hypoxia. Elevated $CO_2$ (hypercapnia) and the resulting low pH of the water typically accompany environmental hypoxia. It is well known that anoxia and hypoxia can result in large numbers of animal mortalities and much research has been conducted on the effects of hypoxia on animals, but the effects of concomitant changes in water $CO_2$ and pH have been less thoroughly studied (Burnett, 1997).

The increased occurrence of hypoxia during the summer, and the high number of summer oyster mortalities attributed to disease, led us to investigate how environmental hypoxia may alter the oyster's ability to defend itself against infection. More specifically, we were interested in examining the oyster's defense system to find out whether it is negatively affected by exposure to environmental hypoxia and to the hypercapnic, low pH conditions that typically accompany hypoxia.

Oysters have a relatively primitive defense system consisting of both cellular and humoral factors (Adema et al., 1991a; Anderson, 1996a). An important role in the defense system is played by phagocytic hemocytes, which can be found throughout the hemolymph and tissues of an oyster (Adema et al., 1991a; Anderson, 1996a). As early as 1950 it was known that hemocytes from *C. virginica* could phagocytose foreign particles and remove them from tissues (Stauber, 1950). Although phagocytosis alone is efficient at removing foreign particles from the tissues, one of the most important mechanisms for cellular killing may be the production of reactive oxygen intermediates (ROIs). When a hemocyte comes in contact with a foreign particle, it phagocytoses it. At the initiation of phagocytosis, hemocytes produce and release ROIs (Adema et al., 1991b). In oysters, hemocytes are thought to produce ROIs including $O_2^-$, $H_2O_2$ (Anderson et al., 1992) and, primarily, HOCl (Austin and Paynter, 1995). These ROIs kill invading cells by inflicting extensive damage, including membrane damage, DNA breakage, enzyme inhibition and amino acid oxidation (Afanas'ev, 1991; Anderson et al., 1992).

For this study we first established the *in vivo* hemolymph conditions (pH, $CO_2$ and $O_2$) when oysters are held in well-aerated normoxic water and hypoxic water. Oyster hemocytes were then incubated in the same pH, $CO_2$ and $O_2$ conditions found *in vivo* during normoxia and hypoxia and the production

of reactive oxygen intermediates (ROIs) was measured. This was done to determine whether hemocytes produce fewer ROIs when an oyster is exposed to environmental hypoxia. Finally, the individual effects of pH, $O_2$ and $CO_2$ on ROI production by oyster hemocytes were measured.

## Materials and methods

### *Oyster collection*

Oysters *Crassostrea virginica* (Gmelin) (6–10 cm) uninfected with the protozoan parasite, *Perkinsus marinus,* were acquired from the Pemaquid Oyster Company in Waldoboro, Maine, USA. South Carolina oysters were not used in this research because of the high incidence of disease. Most, if not all, oysters in South Carolina are infected with *Perkinsus marinus*, also known as dermo (Dwyer and Burnett, 1996), which can affect an oyster in several ways including a decrease in hemolymph pH (Dwyer and Burnett, 1996) and an increase in ROI production by hemocytes (Anderson et al., 1992). Since we were interested in measuring ROI production as a function of pH and other variables, we used uninfected oysters to avoid the complicating effects associated with *P. marinus*. The oysters were packed in ice and shipped overnight to the Grice Marine Laboratory, where they were held in filtered, aerated natural sea water (25 °C, 25 ‰ salinity, pH 7.8–8.0) for at least 4 days.

### *Hemolymph variables*

Oysters were prepared for hemolymph sampling by drilling a small hole in the shell (1.6 mm in diameter) over the adductor muscle. A small patch of dental dam, glued over the hole with cyanoacrylate glue, served as a self-sealing sampling port.

Hemolymph was sampled from oysters held in well-aerated, or ‘normoxic’, sea water at 25 °C, 25 ‰ salinity and pH 7.8–8.0. Hemolymph was also sampled from oysters held for 2 days in hypoxic and hypercapnic water similar to that found in estuaries (25 °C, $P_{O_2}$=4.0–6.7 kPa, pH 7.1–7.4). In addition, hemolymph pH and $P_{O_2}$ were measured after 1-hour incubations in hypoxic and hypercapnic water (25 °C, $P_{O_2}$=4.7–5.3 kPa, pH 7.1) to determine very short-term effects on hemolymph variables.

Approximately 0.5 ml of hemolymph was drawn anaerobically from the adductor muscle of each oyster using a 1.0 ml syringe and a 27-gauge needle. Measurement of variables was made immediately using instruments thermostated to 25±0.1 °C. Hemolymph $P_{O_2}$ was determined with a Radiometer PHM pH/blood gas monitor and $P_{O_2}$ electrode. Hemolymph pH was determined with a Radiometer (BMS2 Mk2 Blood Micro System) capillary pH electrode and PHM pH/blood gas monitor. Hemolymph $P_{CO_2}$ was determined using the Astrup method (Astrup, 1956).

### *Hemocyte collection*

Each oyster was used for only one experiment. To collect hemocytes, the shell of an oyster was notched and all mantle fluid was drained. A 5.0 ml syringe with a 27-gauge needle was inserted into the adductor muscle and hemolymph sampled. Approximately 4–8 ml of hemolymph were sampled from each oyster and immediately placed on ice to prevent hemocyte clumping. The hemolymph was placed in 1.5 ml microcentrifuge tubes and centrifuged at 2,200 ***g*** at 10 °C for 10 min. Pellets containing the hemocytes were removed, pooled and resuspended in 1.0 ml of cell support medium (see below). The hemocyte concentration was then determined by counting cells directly with a hemocytometer. One million hemocytes were required for each assay. For most experiments, hemocytes from more than one oyster were pooled to obtain enough cells to perform an assay.

### *Hemocyte preparation*

The cell support medium (CSM) consisted of 94.5 % filtered (0.2 μmol l$^{-1}$) sea water (25 ‰), 5.0 % fetal bovine serum, 0.5 % antibiotic/antimycotic (10 000 i.u. ml$^{-1}$ penicillin, 10 mg ml$^{-1}$ streptomycin and 25 μg ml$^{-1}$ amphotericin B in 0.9 % NaCl), 0.3 % Hepes buffer and 0.1 % D-glucose. The pH of the CSM was adjusted with NaOH or HCl, according to the specific requirements of an experiment. For experiments on a pH-specific, $O_2$-specific or $CO_2$-specific effect, all variables were held constant at pH 7.6, $P_{O_2}$=20.7 kPa or $P_{CO_2}$<0.07 kPa, except the variable in question. The CSM was incubated under the desired gas pressures for at least 3 hours prior to use, in order to ensure complete equilibration. Gasses were mixed and delivered humidified using Wöstoff gas mixing pumps.

### *Reactive oxygen intermediate production measured by chemiluminescence*

Reactive oxygen intermediate (ROI) production by hemocytes was measured by quantifying chemiluminescence (CL) augmented with the luminescent probe luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) (Van Dyke et al., 1977; Trush et al., 1978; Anderson, 1996a). Luminol augments CL from a variety of ROIs including $H_2O_2$, $OCl^-$ and $O_2^-$ (Anderson, 1996b; Afanas’ev, 1991). Luminol was prepared according to the method of Scott and Klesius (1981) and stored in 1.0 ml portions at −20 °C for no more than 1 week prior to use.

Liquid scintillation vials (10 ml) were used as the experimental chamber. $10^6$ cells were added to each scintillation vial and the volume adjusted to 2.375 ml with the appropriate CSM. Serum stoppers were fitted to each vial, providing a port that was used to flush the gas space in the vial with the desired combination of $O_2$, $CO_2$ and $N_2$. In addition to vials being prepared for measurements of CL under normoxic and hypoxic conditions, a vial with CSM at pH 7.6 and containing ambient room air ($P_{O_2}$=20.7 kPa, $P_{CO_2}$<0.07 kPa) was also prepared. The CL of hemocytes in this vial was used for comparison purposes in statistical tests. All procedures were done at 25 °C and after this point were carried out under dim red illumination to prevent the reaction of luminol with light. Luminol (0.025 ml of 1.0 mmol l$^{-1}$, adjusted to pH 7.6) was injected into each vial and background CL was recorded for at least 15 min or until a stable

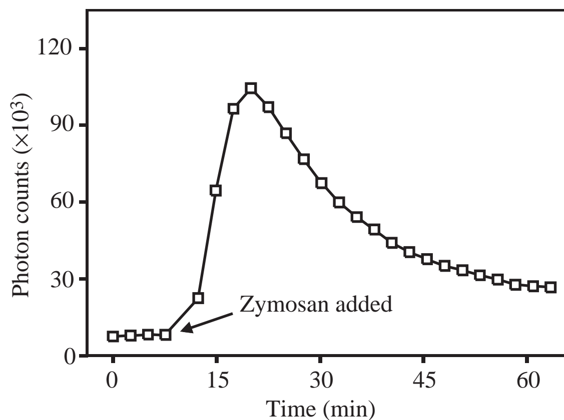


Fig. 1. The profile of increasing photon counts (chemiluminescence) seen after stimulation with zymosan represents an increase in reactive oxygen intermediate production by hemocytes. See Materials and methods for details.

background count was seen. Measurements were carried out in a Wallac 1414 liquid scintillation counter programmed for single photon monitoring at 10 s intervals. A suspension of heat-killed, washed yeast cells (zymosan, Sigma Chemical Company) in CSM was used to induce ROI production by the hemocytes. Each vial was injected with 0.1 ml of the zymosan suspension and CL was measured for approximately 50 min. The total CL of each vial was calculated as the area under the curve after zymosan injection, using the last background count before injection as the baseline for the curve (Fig. 1).

### *Statistical analysis*

Each experiment generated paired data representing the response, or total ROI production, in air, normoxia and hypoxia. For each experiment, data from normoxic and hypoxic treatments were normalized to the response in air. All normoxia and hypoxia data were then compared with a one-way, paired *t*-test. This method also applies to $CO_2$-specific and $O_2$-specific experiments, where $CO_2$ or $O_2$ was varied according to levels found in air, normoxic hemolymph or hypoxic hemolymph, while all other variables were held constant. For pH-specific tests a one-way, paired *t*-test was used on data that were not normalized.

### *Effects of pH on chemiluminescence*

Luminol-enhanced CL is commonly used in biological experiments to measure ROI production. However, a major concern was that there might be a pH effect on the luminol itself. This possibility was investigated in order to ensure that any change in CL observed at different pH values was not simply due to a pH-specific effect on luminol reactivity. To test for a pH effect on the luminol, the pH-induced change in cellular ROI production was measured using two different methods: luminol-enhanced CL (described above) and cellular $O_2$ uptake due to increased ROI production, i.e. the respiratory burst. The technique of measuring $O_2$ uptake is fundamentally different from that of CL and is unaffected by pH. If both methods measured the same pH-induced change in cellular ROI production, it would confirm that there was no effect of pH on the luminol over the range tested.

*Crassostrea virginica* hemocytes could not be used for these experiments. The amount of ROIs produced by oyster hemocytes is much lower than in many other animals, especially vertebrates. ROI production by oyster hemocytes can easily be measured with luminol-enhanced CL, but is difficult to measure by change in $O_2$ uptake. For this reason, macrophages from the fish *Fundulus heteroclitus* were chosen to determine the effects of pH on ROI production. These estuarine animals were easily obtained and both luminol and $O_2$ uptake methods readily measure the ROI production of *F. heteroclitus* macrophages.

### *Macrophage collection*

For each experiment, one fish was anesthetized with 3-aminobenzoic acid ethyl ester (methane sulfonate salt) (MS222). The area immediately behind the head and above the gills was dissected and the anterior kidney removed. The kidney was placed in a fish physiological saline (FPS) containing 96 ml distilled $H_2O$, 2.00 g polyvinyl pyrrolidone, 0.85 g NaCl, 0.71 g Hepes buffer, 0.27 g glucose, 0.05 g $MgSO_4$, 0.04 g KCl, 0.04 g $CaCl_2$ and 0.03 g $NaHCO_3$. The kidney was gently homogenized in a microcentrifuge tube and the cells were counted using a hemocytometer.

### *Immune response measurement and statistical analysis*

The ROI production by macrophages was compared using the CL method and the oxygen uptake method at pH 7.1 and 7.6. Differences in the response to pH were used to assess the sensitivity of the CL techniques to pH. All experiments were paired. $10^6$ phagocytes were added to scintillation vials containing CSM at pH 7.1 and 7.6. $10^6$ phagocytes were also added to respirometer vials containing CSM at pH 7.1 and 7.6. Luminol (0.025 ml of 1.0 mm $l^{-1}$) was added to all vials and background luminescence was measured as previously described. A YSI model 53 meter with a YSI model LN1532 probe was used to determine $O_2$ uptake. Output from the $O_2$ meter was recorded on a Sable Systems Data Acquisition System. The background level of respiration consisted of the pre-stimulation rate of $O_2$ uptake. After 15 min, 0.2 ml of $6\times10^{-5}$ g ml$^{-1}$ phorbol myristate acetate (PMA) was added to all vials to stimulate ROI production. PMA is a receptor-independent agonist, which activates ROI production (Leblebicioglu, 1996), and was used because it resulted in higher levels of ROI production than were attained with other stimulants, such as zymosan. The lower levels of ROI production achieved with stimulants such as zymosan were more difficult to measure using the $O_2$ uptake method.

In the respirometry tests, the baseline $O_2$ uptake rate consisted of uptake due to cellular respiration of all cell types within the sample (primarily red blood cells and phagocytes) and any $O_2$ uptake from background ROI production. After PMA injection, an increase in the rate of $O_2$ uptake results from an increase in $O_2$ demand due to ROI production (Fig. 2)

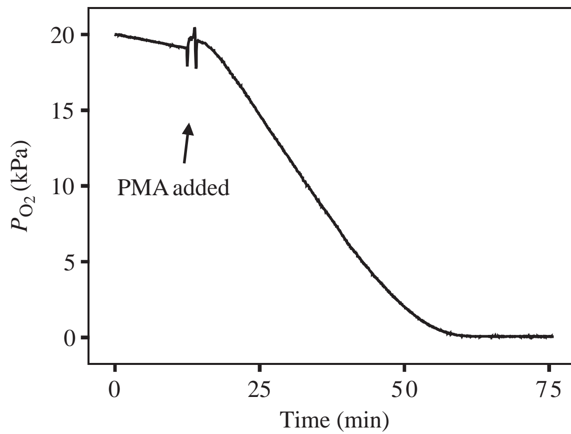


Fig. 2. Tracing of oxygen uptake by *Fundulus heteroclitus* macrophages measured as oxygen depletion in a closed respirometer. Oxygen depletion is shown before and after reactive oxygen intermediate production is stimulated with phorbol myristate acetate (PMA).

(Sharp and Secombes, 1993; Itou et al., 1996). Each assay began with 20.7 kPa $O_2$ and the response was calculated as the maximum rate of $O_2$ uptake after PMA injection minus the baseline $O_2$ uptake rate. Since the response in the respiratory tests was calculated using the peak rate of $O_2$ uptake, the response for the CL tests was calculated using only the peak rate of CL. This method of calculating CL response was employed in order to use peak response measurements in both test types. The percentage changes in ROI production between pH 7.1 and 7.6 using the two different techniques were compared with a two way *t*-test.

## Results

### In vivo *hemolymph variables*

Hemolymph $P_{O_2}$ declined significantly ($P<0.0001$) from 4.9 kPa in oysters held in well-aerated, or 'normoxic' water ($P_{O_2}$=20.0–20.7 kPa, pH 7.8–8.0) to 1.6 kPa after 2 days in hypoxic water ($P_{O_2}$=4.0–6.7 kPa, pH 7.1–7.4 over the incubation period) (Table 1). Hemolymph $P_{CO_2}$ was 0.33 kPa in normoxic conditions and 0.45 kPa in hypoxia ($P$=0.0739). Hemolymph pH decreased ($P<0.0001$) from 7.6 to 7.1 as a result of hypoxia. Hemolymph $P_{O_2}$ in oysters held for 1 hour in hypoxia was similar, but not identical, to those of oysters held in hypoxia for 2 days (Table 1), indicating that the *in vivo* variables respond quickly to changes in water quality. Hemolymph pH was somewhat lower ($P$=0.0042) in oysters held in hypoxia for 1 hour than it was in oysters held in hypoxia for 2 days (Table 1). This may be due to pH compensation by the oysters after a longer period in hypoxia.

### *Hypercapnic hypoxia*

Oyster hemocytes held under conditions approximating hemolymph in animals exposed to hypoxia ($P_{O_2}$=1.47 kPa, $P_{CO_2}$=0.53 kPa, pH=7.1) produced 33 % as much CL as hemocytes held in normoxic hemolymph conditions ($P_{O_2}$=5.2 kPa, $P_{CO_2}$=0.27 kPa, pH 7.6) (Fig. 3). The decrease in ROI production due to conditions simulating hypercapnic hypoxia is significant ($P<0.0001$, $N$=10, one way, paired *t*-test).

Table 1. *Hemolymph variables from oysters in well-aerated (normoxic) and hypoxic water*

| | Hemolymph variables | | |
|---|---|---|---|
| | pH | $P_{O_2}$ (kPa) | $P_{CO_2}$ (kPa) |
| Well-aerated, normoxic water | 7.8–8.0 | 20.0–20.7 | <0.07 |
| Mean ± S.E.M. | 7.55±0.03 | 4.95±0.27 | 0.33±0.04 |
| *N* | 9 | 9 | 9 |
| Hypoxic water | 7.1–7.4 | 4.0–6.7 | approx. 0.4 |
| (1 hour incubation) | | | |
| Mean ± S.E.M. | 6.90±0.05 | 1.10±0.21 | – |
| *N* | 7 | 13 | |
| (2 days incubation) | | | |
| Mean ± S.E.M. | 7.12±0.02 | 1.57±0.10 | 0.45±0.07 |
| *N* | 7 | 6 | 7 |

Oysters were exposed to hypoxic water for 1 hour or for 2 days before variables were measured. See text for details.

### *pH-specific effects*

Oyster hemocytes held at pH 7.1 and stimulated by zymosan produced 48 % as much CL as those held at pH 7.6, while $P_{O_2}$ and $P_{CO_2}$ were held constant (Fig. 4A). The decrease in CL due to the change in pH alone is significant ($P$=0.0052, $N$=10, one way, paired *t*-test).

### *$O_2$-specific effects*

Stimulated oyster hemocytes held at the hypoxic $P_{O_2}$ of 1.47 kPa produced 54 % as much CL as those held at the normoxic $P_{O_2}$ of 5.2 kPa, while $P_{CO_2}$ and pH were held

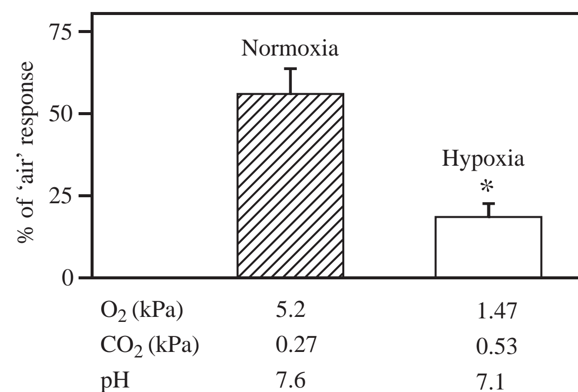


Fig. 3. Reactive oxygen intermediate (ROI) production by oyster hemocytes held in conditions simulating those found in vivo during oyster exposure to well-aerated conditions (normoxia) and to moderate hypoxia. Normoxic and hypoxic ROI production is expressed as a percentage of ROI production by hemocytes held in air. Values are means ± S.E.M. *A significant difference from the response in normoxia ($P<0.0001$, $N$=10).

constant (Fig. 4B). The decline in CL due to oxygen alone is significant ($P$=0.0125, $N$=10, one way paired $t$-test).

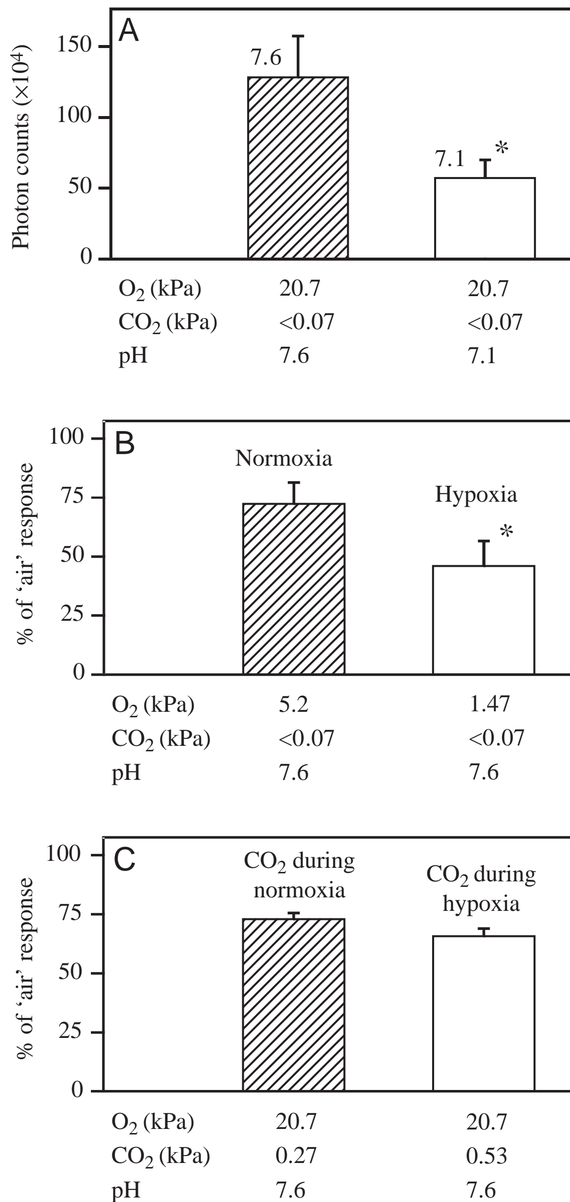


Fig. 4. Effects of pH, oxygen and carbon dioxide on hemocyte ROI production. (A) $CO_2$-independent effects of pH on the ROI production by oyster hemocytes. (B) The effects of oxygen on ROI production by oyster hemocytes incubated in conditions simulating oxygen pressures in 'normoxic' oyster hemolymph and hypoxic oyster hemolymph. Normoxic and hypoxic ROI production are expressed as a percentage of ROI production by hemocytes held in the oxygen pressure of ambient air. (C) The effects of $CO_2$, independent of pH, on ROI production by oyster hemocytes incubated under conditions simulating *in vivo* $CO_2$ levels during well-aerated conditions (normoxia) and moderate hypoxia while all other variables were held constant. Control hemocytes held at a $P_{CO_2}$ of <0.07 kPa were used to normalize data. All values are means ± S.E.M. ($N$=10). *Significant differences ($P$<0.05) (and see text). There was no difference between ROI production at normoxic and hypoxic $CO_2$ levels.

### *$CO_2$-specific effects*

Stimulated oyster hemocytes held at the hypercapnic 'hypoxic' $P_{CO_2}$ of 0.53 kPa produced no less CL ($P$=0.1218, $N$=10, one way paired $t$-test) than those held at the normoxic $P_{CO_2}$ of 0.27 kPa, while $P_{O_2}$ and pH were held constant (Fig. 4C).

### *Effects of pH on chemiluminescence assay*

When stimulated with phorbol myristate acetate (PMA), ROI production by macrophages of *F. heteroclitus* was insensitive to pH. Two different methods of measuring ROI production, $O_2$ uptake and luminol enhanced CL were used and both yielded similar results (Fig. 5). These tests were intended to reveal any direct effect of pH on luminol reactivity by comparing the pH-induced changes in ROI production measured with the two methods. There was no difference in the results obtained by the two methods, indicating that there was no pH effect on the luminol itself. If luminol-enhanced CL had shown a large decrease in ROI production at lower pH values, for example, while respirometry did not, a decrease in luminol reactivity at lower pH would have been indicated. Such a decrease in luminol reactivity would have meant that our results overestimated the measured effects of pH on ROI production, because of a direct effect of pH on luminol. An increase in luminol reactivity would have meant that our results underestimated the severity of the pH-specific effect on oyster hemocytes. All experiments were paired and the results compared using a two-way, paired $t$-test ($P$=0.2099, $N$=7).

## Discussion

Oysters use a variety of methods to defend themselves against infection, including both cellular and humoral factors (Adema et al., 1991a; Anderson, 1996a). Lysozyme, lectins and other factors are produced by cells and then released into

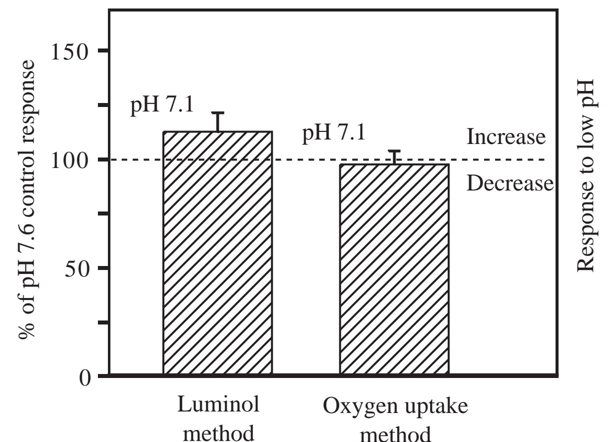


Fig. 5. Reactive oxygen intermediate (ROI) production by PMA-stimulated *Fundulus heteroclitus* macrophages as measured by luminol-enhanced chemiluminescence and oxygen uptake. The response at pH 7.1 is shown as a percentage of the response at pH 7.6. Values are means ± S.E.M. ($N$=7).

the hemolymph (Adema et al., 1991a; Anderson, 1996a). Although phagocytosis is clearly an important cellular defense mechanism for oysters, one of the most important mechanisms for cellular killing may be the production of reactive oxygen intermediates by hemocytes. These ROIs are powerful antibacterial and antiviral agents as well as killers of protozoa (Klebanoff, 1968; Babior et al., 1973; Babior, 1984; Adema et al., 1991a; Marodi et al., 1991; Anderson et al., 1992). Although ROI production in invertebrate hemocytes has not been as well studied as in mammals, it is believed to occur by similar mechanisms (Adema et al., 1991b; Anderson, 1996a).

Hemocytes of *C. virginica* produce ROIs (Adema et al., 1991a; Anderson et al., 1992) and some information is available on their responses to hypoxia. Alvarez et al. (1989, 1992) found that hypoxia has no effect on oyster hemocyte viability, rate of phagocytosis or hemocyte size, but they found that exposure to environmental hypoxia decreases the hemocyte concentration in oysters. While the effects of hypoxia on oysters have not been examined in great depth, there are many examples of the effects of hypoxia and low pH on ROI production by human and other vertebrate cells. Although these studies were done on distant evolutionary cousins of the oyster, they provide some insight into why the hemocytes respond as they do.

Recent studies by Bramble and Anderson (1997, 1998, 1999) indicate that the hemocytes of *C. virginica* produce only a fraction of the ROIs that are produced by fish macrophages. Furthermore, Bramble and Anderson (1998) showed that bacterial antioxidants may suppress oyster hemocyte CL. More recently Bramble and Anderson (1999) showed that the bactericidal activity of *C. virginica* hemocytes does not involve ROI production against several different kinds of bacteria. These studies raise significant and important questions about the involvement of ROI production in the oyster defense system. Clearly the effects of hypoxia on oyster defense systems as well as bacterial antioxidant mechanisms require further investigation.

Overall, hypoxia and the conditions that accompany hypoxia, i.e. elevated $P_{CO_2}$ and low pH, clearly reduce production of ROIs by hemocytes (Fig. 3). This effect occurs when oyster hemocytes are exposed simultaneously to the pH, $O_2$ and $CO_2$ pressures they experience *in vivo* during periods of moderate hypoxia. In the present work the effects of the individual variables ($O_2$, $CO_2$ and pH) were evaluated separately. A reduction of oxygen, independent of $CO_2$ and pH, reduces ROI production (Fig. 4). Hemocytes held at a hypoxic $P_{O_2}$ of 1.47 kPa produced 54 % as much CL as those held at the normoxic hemolymph $P_{O_2}$ of 5.2 kPa. Although other researchers have worked at somewhat different oxygen pressures and on other animals, a similar trend has usually been found. Edwards et al. (1984) found that ROI production by rat neutrophils decreases progressively as the level of oxygen drops below 3 % (approximately 3.2 kPa) and is at half the maximal at 1 % oxygen. Similarly, ROI production by human neutrophils decreases as oxygen levels fall to 1 % and reached negligible levels at oxygen concentrations of less than 0.25 % (Gabig et al., 1979). Zulueta et al. (1995) reported that, after 2 h of exposure to 3 % oxygen, bovine pulmonary artery endothelial cells released 70 % less $H_2O_2$ than they did at 20 % oxygen and 96 % less at 0 % oxygen. Kinnula et al. (1993) found that release of $H_2O_2$ by bovine endothelial cells in culture decreased by approximately 30 % when oxygen concentration was dropped from 10 % to 5 %. Finally, Feldman et al. (1990) found that superoxide production by mouse macrophages at 10 % oxygen was less than half of what it was at 15 % oxygen.

Over 100 mammalian oxidases use oxygen as a substrate and terminal electron acceptor (Keevil and Mason, 1978). Superoxide results from a one-electron reduction of $O_2$ and $H_2O_2$ results from a two-electron reduction. These oxidases vary widely in oxygen affinity (De Groot and Littauer, 1989). It is possible that the mechanism for oxygen-related reductions in ROI production involves an oxygen-dependent decrease in oxidation and/or autoxidation reactions (De Groot and Littauer, 1989; Yang and Block, 1995).

The 52 % decline in ROI production with a 0.5 pH decrease (Fig. 4) is consistent with results obtained using vertebrate neutrophils. For example, Hackam et al. (1996) found that decreasing the pH from 7.4 to 6.8 caused a significant reduction in superoxide production by exudative rat neutrophils. When Leblebicioglu et al. (1996) reduced the pH from the normal physiological pH of 7.2 to 6.7 there was more than a 33 % reduction in the amount of superoxide produced by human neutrophils. Gabig et al. (1979) demonstrated that a change in pH from 7.5 to 6.2 caused an 80–90 % decrease in the $O_2$ production by human neutrophils. Newberger et al. (1980) found that superoxide production by human granulocytes was maximal at pH 7.5 and decreased with pH. More recently, Geffner et al. (1993) found that the respiratory burst of polymorphonuclear leukocytes was lower in acidic environments.

The mechanisms of the pH-specific effects on ROI production may be related to the NADPH oxidase system, which mediates the production of ROIs. In a cell-free system, NADPH oxidase activation is reported to be optimal between pH 6.8 and 7.9 (Tauber and Goetzl, 1979) and between pH 7.0 to 7.5 (McPhail et al., 1985). At pH values below 6.8 the NADPH system loses activity markedly (Swallow et al., 1993). Hackam et al. (1996) found that exudative rat neutrophils suffer a decrease in both the internal pH and in superoxide production when the external pH was dropped from 7.4 to 6.8. At external pH 6.8 the internal pH of exudative cells dropped from 7.25 to 6.6 (Hackam et al., 1996). Several researchers have found that cytoplasmic acidification impairs the ability of neutrophils to generate a respiratory burst (Simchowitz, 1985; Grinstein et al., 1991; Swallow et al., 1993). Something similar might occur in oyster hemocytes. The intracellular pH of oyster hemocytes becomes highly acidic during phagocytosis (Beaven and Paynter, 1999), but it is not known how intracellular hemocyte pH responds to hemolymph pH. As the pH of the hemolymph drops, the internal pH of the hemocyte may also decrease, and such a pH decrease might lower the

activity of many enzymes, including NADPH oxidase. Since many cell functions are directly or indirectly involved in hemocyte synthesis of ROIs, it is possible that a change in internal pH could disrupt one or more pathways, resulting in a reduction in ROI production.

Our results suggest another possible cause. ROI production by the phagocytes of *Fundulus heteroclitus* stimulated with zymosan demonstrate a strong pH dependence (unpublished observations), but when we examined ROI production by the same cell type stimulated with PMA there was no effect of pH on ROI production (Fig. 5). Some investigators have reported a pH effect when using PMA (Newberger et al., 1980); however, our results are similar to those found by Leblebicioglu et al. (1996) with human neutrophils. When using fMet-Leu-Phe (fMLP) with the neutrophils, Leblebicioglu et al. (1996) found a significant effect of pH on superoxide production. However, when using PMA there was relatively little pH effect. Unlike zymosan or fMLP, PMA is a receptor-independent agonist, which fully activates the NADPH oxidase system. As first suggested by Leblebicioglu et al. (1996), these results may indicate that the decreased external pH is affecting receptors or calcium channels on the surface of the cells. Even if the ability of the cells to produce ROIs is not directly affected by the decrease in pH, the ability of the cells to turn on the system could be reduced. Although we do not know whether receptors or calcium channels are actually implicated, the PMA results suggest that the pH effect may be occurring before the NADPH system is even activated. This is because PMA, which produces no pH effect, bypasses this system.

Luminol tests using *Fundulus heteroclitus* showed that there was no pH effect on luminol itself over the range used (7.1–7.6). However, preliminary experiments using *F. heteroclitus* macrophages suggested that at lower pH, such as 6.5, pH may begin to decrease the reactivity of luminol. For experiments outside the pH range used here, pH-specific effects on luminol should be considered.

There was no significant effect of carbon dioxide on ROI production independent of pH (Fig. 4). The range of $CO_2$ pressures used for our experiments was relatively narrow and these results do not mean that $CO_2$ would have no effect at the higher pressures that may be encountered during periods of severe hypercapnic hypoxia. Even if future work reveals a $CO_2$-specific effect on ROI production, it would be a rare occasion in which environmental $CO_2$ levels would be high enough to produce the degree of ROI inhibition already seen with low oxygen and pH.

Although this research produced highly significant results, hemocytes were not exposed to the extremes that they may experience during periods of severe hypoxia. Oysters were incubated under relatively moderate hypoxia and pH conditions. It is not uncommon to find periods of hypoxia and low pH more severe than those used for this research (Cochran and Burnett, 1996). Consequently, oysters may suffer frequently from even greater decreases in their ability to produce ROIs than were seen here. Since the oysters used in these experiments were subtidal and from Maine, the physiology of native South Carolina oysters during air exposure was not examined. Dwyer and Burnett (1996) found that hemolymph pH and oxygen pressures in South Carolina oysters exposed to air were similar to hypoxic oysters used in the present study.

Although we found that oxygen levels affect ROI production, Alvarez et al. (1989, 1992) found no effect of anoxia or hypoxia on the rate of phagocytosis and particle clearance times in oysters. Taken together, these works indicate that simply measuring the rate of phagocytosis to gauge the defense response of oyster hemocytes does not provide a complete picture. We also found that the response and the recovery times of hemocytes were rapid. When normoxic hemocytes were exposed to hypoxic conditions, or vice versa, ROI production was affected by the start of CL measurements, i.e. occurred within 15 min. It would be interesting to see whether the response of hemocytes of oysters held in chronic hypoxia are different from those subjected to the acute exposures used in this study.

Two main conclusions may be drawn from this work. First, it suggests that hypoxia and the accompanying low pH affect the physiology of oysters in a negative way by reducing their ability to defend themselves against infection. As shown by this research, the reduction in the ROI production of oysters occurs as a result of even moderate hypoxia and is undoubtedly exacerbated during seasonal periods of more severe hypoxia. Such a decrease in resistance may be a major contributor to the incidence of disease in oysters. Second, it demonstrates how important it is to take into account *in vivo* oxygen and pH levels when doing research on cellular response. However, what these results mean with respect to the survival of the oyster is not clear. Oysters may suffer from mass disease-related mortalities or might simply have a seasonal decrease in fitness due to hypoxia.

We would like to acknowledge the technical assistance of Kim Boleza, Karen Burnett, Chris Milardo, Tina Mikulski and Libby Willson. This research was supported by South Carolina Sea Grant R/ER–14, Sigma Xi and the Slocum-Lunz Foundation, and is contribution 156 to the Grice Marine Laboratory.

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